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Analysis of Flavonolignans in Dried Fruits of *Silybum marianum* (L.) Gaertn from Iran

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Abstract: The content and composition of main component of Silymarin in dried fruits of *Silybum marianum* L. Gaertn collected from different environmental conditions of Iran were analyzed by TLC and HPLC method and compared with those from Hungarian fruits. For determination of presence of flavonolignans, samples were analyzed by TLC and five bands were revealed related to silybin, isosilybin, silychristin, silydianin and taxifolin. The amount of flavonolignans was determined by HPLC method and two pairs of diasteroisomeric silybin (silybin A, silybin B, isosilybin A, isosilybin B) were separated. The results showed that silymarin amount in fruits of different areas were very different from each other and the highest amount of silymarin content were in fruits of Borazjan (south-west of Iran) and Rudbarak (north of Iran) (27.1 and 24.6 mg g^{-1} dry Wt.) The major flavonolignan of this sample (Borazjan) were silybin B and isosilybin B. The content of silymarin in fruits of plants that grown under greenhouse conditions (3.3 mg g^{-1} dry Wt.) was much lower than amount of silymarin in fruits of other areas and Hungarian fruits (22.7 mg g^{-1} dry Wt.).

Key words: *Silybum marianum* (L.) Gaertn, flavonolignans, Silymarin, Silybin, HPLC, TLC

INTRODUCTION

The milk thistle (*Silybum marianum* L. Gaertn) is an annual or biannual herbaceous plant that is widespread in temperate American countries, Australia and Mediterranean climate[1,2] and recommended to be used in traditional European and Asiatic medicine, mainly for treatment of liver disorders and it is presently one of the most commonly used medicinal plants worldwide[3]. Their hepatoprotective activities seems to be based on antioxidant properties[4], which prevent lipid peroxidation and cell membrane destruction, as well as stimulation of protein biosynthesis and acceleration of cell regeneration in damaged livers[3]. The active component of dried fruits extract of milk thistle, consisting of various flavonolignans and isoflavanoid taxifolin (TXF), is known as silymarin. The flavonolignans in silymarin include silybin A (SBN A), silybin B (SBN B), isosilybin A (ISBN A), isosilybin B (ISBN B), silydianin (SDN) and silychristin (SCN) (Fig. 1)[5,6]

Several studies have shown that SBN is the main component of silymarin, both quantitatively[7] and therapeutically, although SCN and SDN play antioxidative activity. Krecman *et al.*[8] suggested that SBN is even more effective when associated with other constituents, probably because the availability of former compound is lower when alone than when it forms part of the silymarin complex [1].

It is now accepted that there is an integral interaction between plants and their environment and that species specific secondary metabolites are important constituent in this interaction[9].

Flavonoid natural products have diverse functions in development and interactions with the environment[10].

It is clear understands that how environmental factors affects on phytomedicinal production toward optimizing field growth conditions for maximal recovery of phytomedicinal chemicals[11].

In the present research we analyzed the production of silymarin and its active components in dried

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fruits of *Silybum marianum* collected from 13 areas of Iran (Table 1) and compared with fruits from Hungary.

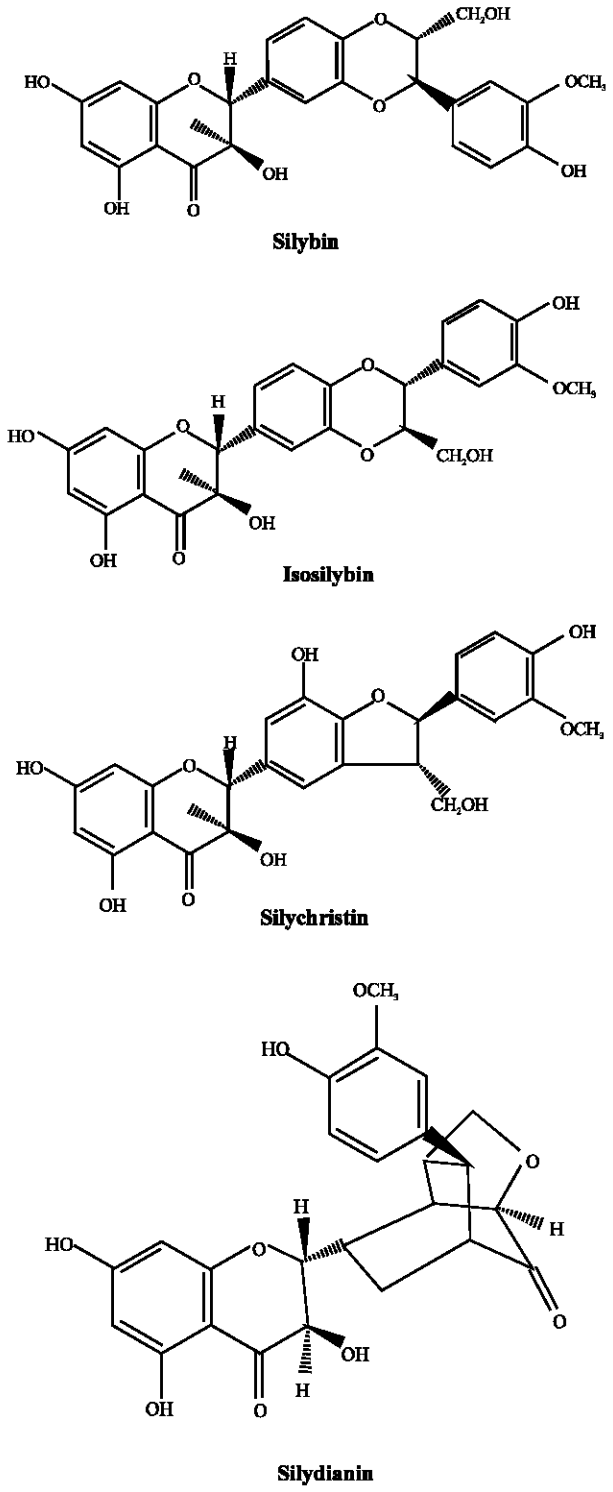

Fig. 1: Most important components of the silymarin mixture

MATERIALS AND METHODS

Extraction and Isolation: During Jun 2003, dried fruits of Silybum marianum were collected from 13 area of Iran. (North, West and South-West) (Table 1) and Hungarian fruits were supplied by Institute of Medicinal Plants. Plants were cultivated in Karaj at greenhouse conditions with a 18 h photoperiod.

The flavonolignans were extracted according to Alikaridis *et al.*[3] and Quaglia *et al.*[12] The methanolic solution was concentrated to a dry residue. The residue was dissolved in 50 ml of methanol and kept at 4°C.

TLC analysis: The flavonolignans detected by Thin Layer Chromatography (TLC) according to Wagner *et al.*[13] Five bands were revealed under UV light at 366 nm with different colors.

SBN, ISBN, SCN, SDN and TXF were identified by comparison of R_f to standards of SCN, SDN supplied by phytolab, SBN, TXF and a standard mixture of silymarin provided by Sigma chemicals.

HPLC analysis: The amount of flavonolignans was determined by High Performance Liquid Chromatography (HPLC) on a knauer liquid chromatography equipped with a knauer injector with a 20 μL loop, a Nucleosil C18 5 μ (250×4.6 mm) column, knauer K2600A UV detector and Chromgate software for peak integration. Mobil phase consisted of the solvents, with a gradient program (Table 2). All solvents and chemicals were of HPLC grade (Merck). The elution time and flow rate were 30 min and 1 mL min^{-1} and peaks detected at 288 nm[12].

Identification was achieved by comparison of retention times (R_t) to standards of SCN, SDN, SBN, TXF and a standard mixture of silymarin.

Quantification of these metabolites, expressed in mg g^{-1} of dry weight, was accomplished using a known concentration of standard and peak areas. A standard solution of SBN was freshly prepared. With dilution of this methanolic solution a series of solution at different concentration were prepared and used as standard. The data obtained from the analysis of each solution allowed to plot a calibration curve showing a good linearity (correlation coefficient = 0.999). The quantitative data obtained from the HPLC samples analysis have been summarized in Table 4.

All experimental analyses were carried out on a minimum of three independent samples for each region and three replications of each sample were assayed. Statistical significance was calculated using Duncan test for unpaired data ($\alpha \leq 0.05$) and ANOVA method was used

for comparisons of means. Statistical analysis was made by SAS software for windows (Version 6.2).

RESULTS

Methanolic extract of dried fruits, for presence of flavonolignans were analyzed by TLC according to Wagner *et al.*[14] . After treatment with NP/PEG reagent, chromatograms were revealed after 15 min in UV-366 nm (Table 3). TXF (R_f = 0.45), with orange fluorescent band, green-yellow fluorescent bands of SCN (R_f = 0.40) and SDN (R_f = 0.55), dark yellow fluorescent bands of SBN A and SBN B (R_f = 0.6) and light yellow fluorescent band for ISBN A and ISBN B (R_f = 0.65). The fluorescent zones above SBN were due to dehydro-derivatives of SBN and ISBN (R_f = 0.65). With this method SBN and ISBN moved separately and SDN was between TXF and SBN.

HPLC analysis carried out and TXF (R_t = 2.50), SCN (R_t = 3.50), SDN (R_t = 4.00), SBN A (R_t = 9.70), and SBN B (R_f = 10.50), ISBN A (R_t = 11.30), ISBN B (R_f = 12.13) were isolated (Table 3). Two pairs of diasteroisomeric flavonolignans, silybin A, silybin B, isosilybinA, isolilybinB were successfully separated with this method.

Data from the analyses of silymarin amount by HPLC method (Table 4) showed that amount of silymarin in fruits collected from Borazjan[12], Rudbarak[3] and Hungry[16] were much higher than from other areas. There was no significant difference between silymarin means in Valyabad with different altitudes (1 and 8). It is interesting that the content of silymarin in fruits of plants that grown in karaj under greenhouse conditions[15] was much lower than amount of silymarin in fruits of Hungary.

Table 1: Sampling for determination of flavonolignans of dried fruits of *Silybum marianum*

Origin of fruits	Altitude (m)	Latitude (m)	Longitude (m)	Min. Tem. (Jun.)	Max. Tem. (Jun.)	Min. Hum. (Jun.)	Max. Hum (Jun)
1	1800	36 14	51 18	24	32	61	89
2	1260	36 26	51 20	22	33	60	87
3	1180	36 29	51 7	24	31	58	90
4	1040	36 31	51 15	23	34	57	88
5	830	36 23	51 18	22	32	57	88
6	460	36 27	51 18	22	30	59	90
7	400	36 28	51 21	23	33	59	89
8	100	36 15	51 18	25	30	60	85
9	1560	33 29	48 22	16	37	10	43
10	120	31 20	48 40	25	39	9	21
11	830	29 36	52 32	18	36	18	51
12	110	29 20	51 17	22	26	60	82
13	60	28 59	50 50	29	39	61	93
14	1520	35 52	50 59	22	37	10	55
15	1520	35 52	50 59	20	30	60	65

Dried fruits of 1- 8 collected from Mazandaran, Chaloos, North of Iran ([1]Valyabad,[2] Hezarcham, [3]Rudbarak, [4]Banafshedeh, [5]Valasht, [6]Marzanabad, [7]Hasanabad, [8]Valyabad. 9, collected from Lorestan, Khorramabad, west of Iran. 10, from Khusestan, Ahvaz. 11, from Fars, Kazerun. 12 and 13 from Bushehr, [12]Bushehr and [13]Borazjan, Sought - west of Iran. 14 and 15 plants grown in [14]Karaj and [15]greenhouse condition

Table 2: HPLC gradient solvent program

Time (min)	Methanol (mL min^{-1})	Acetonitril (mL min^{-1})	Water (pH = 2.3 With 10% H_3Po_4)(mL min^{-1})
0:00	22	15	63
7:30	22	15	63
15:00	40	20	40
30:00	22	15	63

Table 3: Determination of R_t and R_f of flavonolignans by TLC and HPLC

Compound	R_t	R_f
TXF	2.5	0.45
SCN	3.5	0.4
SDN	4	0.55
SBN A	9.7	0.6
SBN B	10.5	0.6
ISBNA	11.3	0.65
ISBNB	12.13	0.65

By comparing the flavonolignan content especially silymarin and SBN and since SBN generally is primary active component, fruits of Borazjan[12] from South-west of Iran had the highest amount of silymarin (27.102 mg g^{-1} dry wt.) and the major flavonolignan of this sample is SBN (SBN B 75% and SBN A 25%).

As it can be seeing in Table 4, amount of SBN A in Banafshehdeh[4] and Borazjan[12] and amount of SBN B in Khorramabad[9] and Borazjan[12] are about twice the amount of SBN A and SBN B in Hungarian fruits and were much higher than those from other regions.

Results showed that fruits from Bushehr[13], khorramabad[9] and Hungrian fruits that were grown in Karaj under greenhouse conditions didn't have SBN A whereas fruits of Khorramabad[9] did not have SBN B. Furthermore, amount of SBN B in Borazjan[12] was higher than Banafshehdeh[4].

ISBN B in fruits collected from Borazjan[12] was nearly twice the amount of kazerun[11], Rudbarak [3], Ahvaz[10] and Hungry. Amount of this metabolite in fruits of other regions were much low while amount of ISBN A in Borazjan[12] and Kazerun[11] were much lower. Amount of ISBN A in Rudbarak[3] was much higher than other areas.

As shown in Table 4 amounts of ISBN B, and TXF in Hasanabad[7], Marzanabad[6] and Valasht[5] in comparison with other areas were low but amount of ISBN A in these areas was high.

Amount of SDN in Rudbarak[3] and Borazjan[12] and Amount of SCN in Rudbarak[3] and Hungarian fruits were much higher and amounts of these metabolites in Valasht[5], Hezarcham[2], Banafshehdeh[4], Marzan-abad[6], HasanAbad[7], Valyabad (1 and 8) were low. Amount of SCN in fruits of Ahvaz[10] were high in comparison with other metabolites.

Amount of TXF in Rudbarak[3] was much higher than what was observed in other regions while amount of TXF in fruits from Hungry, Borazjan[12], Ahvaz[10] and Kazerun[11] did not have significant difference with amount of TXF in fruits from Rudbarak[3].

Table 4: Contents of flavonolignans (mg g^{-1} dry Wt±SD) in dried fruits of *Silybum marianum* from different areas of Iran (1-15) and Hungarian fruits (16)

	Contents of flavonolignans							
Sample	TXF	SCN	SDN	SBN A	SBN B	ISBN A	ISBN B	Silymarin
1	0.431±0.09	0.349±0.02	0.318±0.01	1.616±0.07	3.263±0.01	2.742±0.11	0.00	8.728±0.46
2	1.098±0.62	1.012±0.70	0.968±0.64	1.434±0.77	2.569±0.75	4.482±2.18	1.412± 0.75	12.690±6.22
3	3.058±0.08	3.542±0.24	6.062±0.541	0.196±0.11	1.184±0.18	7.114±0.51	3.415±0.25	24.573±1.750
4	0.731±0.04	0.576±0.01	0.849±0.21	3.134±0.07	4.685±0.05	4.168± 0.43	0.341±0.34	12.690±0.36
5	0.846±0.12	0.733±0.26	0.994±0.51	1.319±0.34	2.169±0.52	2.745±0.11	0.941±0.31	14.488±0.65
6	0.783±0.04	0.490±0.01	0.591±0.03	1.034±0.06	1.421±0.09	4.061±0.19	0.00	9.748±0.44
7	0.739±0.01	0.465±0.01	0.532±0.03	1.434±0.49	3.935±0.40	4.140±0.22	0.00	8.382± 0.74
8	0.628±0.02	0.432±0.03	0.391±0.02	0.621±0.31	1.479±0.28	2.178±0.08	1.197±0.06	6.929±0.25
9	1.684±0.25	0.00	4.544±0.68	0.00	7.656±1.18	3.011±0.52	0.339±0.19	17.236±2.51
10	2.379±0.25	2.246±0.70	2.447±0.29	0.881±0.16	2.498±0.51	2.436±1.80	3.341±1.21	16.230±1.34
11	2.227±0.26	0.380±0.38	4.093±0.23	1.447±0.30	3.905±0.74	2.082±1.26	3.811±0.70	17.947±1.27
12	2.513±0.005	0.00	5.776±0.005	2.349±0.02	6.401±0.005	1.443±0.01	8.619±0.03	27.102±0.01
13	1.407±0.01	0.825±0.008	2.258±0.008	0.00	0.654±0.008	2.721±0.01	1.256±0.01	9.124 ±0.008
14	1.778±0.38	0.850±0.31	2.871±0.50	0.00	1.968±1.13	3.751±2.16	0.00	11.219±3.91
15	0.348±0.08	0.387±0.10	0.593±0.11	0.00	0.394±0.10	1.083±0.21	0.491±0.07	3.290±0.69
16	2.517±0.004	3.166± 0.004	4.673±0.01	1.242.±0.08	4.494.±0.08	3.892±0.05	2.759±0.02	22.733±0.01

DISCUSSION

Plants respond flexibly to environmental stimuli that act upon plastic program of development[10] and secondary metabolites can be key players in the interaction between plants and their environment[9]. The present data indicate that, silymarin content in fruits from different environmental conditions of Iran is very different. The comparison of the silymarin and SBN amounts in fruits from greenhouse conditions with fruits from Hungry showed that silymarin and SBN amounts with same genotypes were very different, possibly as a result of correspond to environmental conditions. According to Schmid *et al.*[10] Chalcone synthase (CHS) catalyses the stepwise condensation of three acetyl unite from malonil-CoA with 4-hydroxycinnamoyl-CoA to give naringenin chalcone, which is the first committed step in the branch pathway of phenylpropanoid metabolism specific for flavonoid biosynthesis. CHS is a key metabolic control point[15] and CHS mRNA and enzyme levels are highly regulated during development associated with the tissue and cell type-specific accumulation of flavonoid and in response to environmental stimuli for the synthesis of flavonoids involved in adaptation or protection[10].

Silybin and isosilybin are mixture of diasteroisomers which ratio of SBNA to SBNB is about 47:58[6] and present results showed that in all of samples content of SBN B in fruits was higher than SBN A, especially in fruits of sample 12. ISBN A, which occurred in a ratio of about 77:28 to ISBN B[6], was obtained about 67:32 in fruits of sample 3, 14:85 in sample 12, 68: 31 in sample 15 and 58:41 in Hungarian sample. The peaks of silymarin and TXF accumulation was same and a considerably larger accumulation of silymarin was relative to TXF accumulation and the amount of silymarin decreased as TXF decreased, as would be expected, TXF acts as a precursor to silymarin biosynthesis. There was no significant difference between amounts of silymarin in valyabad with different altitude and amount of silymarin in this area was very low that may be due to various factors including low expression of key enzymes[10].

This study has increased the understanding of flavonolignans products in fruits of *Silybum marianum* from different areas which was previously poorly known but it is also unknown as to the extent to which levels of phytomedicinal chemical production by medicinal plants are determined by genetic potential versus environmental modulation. The use of molecular markers in the characterization of medicinal plant populations for levels of phytomedicinal chemical production could be useful.

The present study provides an experimental basis for horticultural research on medicinal plants has focused on developing the capacity for optimal growth in cultivation.

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